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Research paper

The two *Dictyostelium* autophagy eight proteins, ATG8a and ATG8b, associate with the autophagosome in succession

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ABSTRACT

Autophagy is an ancient cellular pathway that is conserved from yeast to man. It contributes to many physiological and pathological processes and plays a major role in the degradation of proteins and/or organelles in response to starvation and stress. In the autophagic process cytosolic material is captured into double membrane-bound vesicles, the autophagosomes. After fusion with lysosomes, the cargo is degraded in the generated autolysosomes and then recycled for further use.

Autophagy 8 (ATG8, in mammals LC3), a well-established marker of autophagy, is covalently linked to phosphatidylethanolamine on the autophagic membrane during autophagosome formation. Bioinformatic analysis of the *Dictyostelium* genome revealed two *atg8* genes which encode the ATG8a and ATG8b paralogs. They are with around 14 kDa similar in size, 54 % identical to one another and more closely related to the corresponding proteins in fungi and plants than in animals. For ATG8a we found a strong up-regulation throughout the 24 h developmental time course while ATG8b expression was highest in vegetative cells followed by a moderate reduction during early development. Confocal microscopy of fluorescently tagged ATG8a and ATG8b in vegetative AX2 wild-type and in ATG9$^-$ cells showed that both proteins mainly co-localized on vesicular structures with a diameter above 500 nm while those smaller than 500 nm were predominantly positive for ATG8b. In ATG9$^-$ cells we found a strong increase in the relative abundance of ATG8a-positive large vesicular structures and of total ATG8b-positive structures per cell indicating autophagic flux problems in this mutant. We also found that vesicular structures positive for ATG8a and/or ATG8b were also positive for ubiquitin. Live cell imaging of AX2 and ATG9$^-$ cells co-expressing combinations of red and green tagged ATG8a, ATG8b or ATG9 revealed transient co localizations of these proteins. Our results suggest that ATG8b associates with nascent autophagosomes before ATG8a. We further find that the process of autophagosome formation in *Dictyostelium* is highly dynamic. We infer from our data that *Dictyostelium* ATG8a and ATG8b have distinct functions in autophagosome formation and that ATG8b is the functional orthologue of the mammalian LC3 subfamily and ATG8a of the GABARAP subfamily.

1. Introduction

Autophagy is the major lysosomal pathway for the degradation of large cellular components. It is crucial for fundamental biological processes such as development, immunity, cell death and aging, and is also associated with a number of diseases such as cancer, neurodegeneration and the cellular response to invading pathogens (Hanada et al., 2007; Klionsky and Emr, 2000; Shintani and Klionsky, 2004). The process is tightly controlled, induced by starvation and other stresses and can be subdivided into three different types: macroautophagy, microautophagy and chaperone-mediated autophagy (Shintani and Klionsky, 2004). Macroautophagy is the routine turnover of cytosolic components and this process occurs at basal levels in most tissues (Shintani and Klionsky, 2004). It was generally considered as a non-selective degradative pathway but there is increasing evidence that most if not all types of macroautophagy (hereafter autophagy), e.g. pexophagy, the turnover of peroxisomes, ribophagy or mitophagy are selective (Li and Vierstra, 2012; Lippai and Lőw, 2014; Wang and Klionsky, 2003; Weidberg et al., 2011).

The autophagic process is subdivided in the initiation, autophagosome maturation and lysosomal degradation phases. The evolutionarily conserved machinery encompasses more than forty core and accessory autophagy-related (ATG) proteins, many of which have been initially characterized in yeast (Tsukada and Ohsumi, 1993). In the initiation phase the phagophore or isolation

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membrane is formed de novo with lipids from different membrane sources. The newly formed double-membrane structure is expanded, enwraps cytoplasmic macromolecules and organelles and matures into a closed vesicle, the autophagosome. In the final step the outer membrane of the autophagosome eventually fuses with the lysosome (or vacuole in yeast). This gives rise to the auto(phago)lysosome in which the cargo and the inner membrane of the autophagosome are degraded by resident hydrolases (Chen and Klionsky, 2011; Lamb et al., 2013; Mizushima et al., 2002; Wang and Klionsky, 2003).

Two ubiquitin-like conjugation systems are indispensable for autophagosome formation. The first system leads to the formation of a tetrameric complex consisting of two ATG12-ATG5 conjugates bound to an ATG16 dimer (Abounit et al., 2012; Kuma et al., 2002; Mizushima et al., 1999, 2003). The covalent linkage of ATG8/LC3 to the phospholipid phosphatidylethanolamine (PE) is the second ubiquitin-like modification step in autophagy and follows the formation of the ATG12-ATG5/ATG16 complex. In this reaction the cleaved and activated ATG8/LC3-I is transferred by the E2-like enzyme ATG3 to the ATG12-ATG5/ATG16 complex. ATG16 determines the binding site at the pre-autophagosomal structure (PAS) in yeast or the isolation membrane in higher eukaryotes (Fujita et al., 2008; Suzuki et al., 2001). Then ATG12-ATG5 catalyses the lipidation of ATG8/LC3-I with PE (giving rise to LC3-II) through its E3-ligase activity (Hanada et al., 2007). ATG8/LC3-II appears to have a role in membrane closure and is involved in cargo selection by recruiting cargo adaptor proteins, for example p62 and Nbr1, via their ATG8 interacting motif (AIM) or LC3 interacting region (LIR), respectively (Abounit et al., 2012; Birgisdottir et al., 2013; Nakatogawa et al., 2007; Weidberg et al., 2011). The process of cargo selection for degradation is best understood for p62, which can bind ubiquitinated proteins and deliver them through binding to ATG8/LC3-II to the growing autophagosome (Bjorkoy et al., 2005; Abounit et al., 2012). Oligomerization enables p62 to deliver several ubiquitinated proteins to a single ATG8/LC3-II molecule (Abounit et al., 2012).

ATG8/LC3 is associated with the autophagosomal membrane from an early stage till the fusion with the lysosome and it is therefore widely used to monitor autophagy. Functional studies of ATG8/LC3 in higher organisms are complicated by the fact that multicellular animals express two or more ATG8/LC3 paralogs. For example nematodes possess three genes for *atg8* and mammals express seven genes which are grouped into the MAP1-LC3 (microtubule associated protein 1–light chain 3) subfamily with three members (LC3A, B and C) and the GABARAP/GATE-16 (Gamma-aminobutyric acid receptor-associated protein/Golgi-associated ATPase enhancer of 16 kDa) subfamily with four members (Shpilka et al., 2011). The respective precise functions of the seven mammalian ATG8 paralogs are largely unresolved. In contrast fungi possess only a single *atg8* gene while plants and amoebozoa like *Dictyostelium discoideum* usually have two genes, facilitating the analysis of their respective function in autophagy (Kabeya et al., 2004; Shpilka et al., 2011).

The social amoeba *D. discoideum* has become an increasingly important model system for the investigation of autophagy in recent years. In this organism autophagy genes can be easily disrupted and their single or double knock-outs resulted in informative phenotypes. In addition, novel conserved autophagy genes have been discovered and the autophagy machinery is more similar to higher eukaryotes than to yeast (Calvo-Garrido et al., 2010; Calvo-Garrido and Escalante, 2010; King, 2012; Munoz-Braceras et al., 2015; Tung et al., 2010; Xiong et al., 2015). Here we used *D. discoideum* to investigate potentially different functions of its two ATG8 paralogs, ATG8a and ATG8b, during autophagosome formation. We analyzed their developmental regulation and performed co-localization studies of live and fixed cells using strains that ectopically expressed either RFP-ATG8a, GFP-ATG8a and/or RFP-ATG8b in AX2 wild-type cells, ATG9$^-$ cells and in ATG9$^-$ cells re-expressing ATG9-GFP (AAG strain) (Tung et al., 2010). Our results show that ATG8a and ATG8b largely co-localize on vesicles larger than 500 nm, while smaller vesicles were predominantly positive for ATG8b. Live-cell imaging revealed the high dynamics of vesicular structures positive for ATG8a, ATG8b and/or ATG9 and showed that these proteins associate with autophagosomes in succession. Our data suggest that ATG8a and ATG8b fulfill different functions during autophagosome formation and we suggest that ATG8b like LC3 is more important in the early phase while ATG8a like GABARAP has a function in the later phase (Weidberg, Shvets, et al., 2010; Weidberg et al., 2011).

2. Materials and methods

2.1. Dictyostelium *strains and culture conditions*

D. discoideum AX2 was used as wild-type strain. Mutant strains expressing either RFP-ATG8a, RFP-ATG8b, RFP-ATG8a and GFP-ATG8a or RFP-ATG8a and GFP-ATG8b were generated in AX2 or AAG cells (ATG9$^-$ cells expressing ATG9-GFP) (Tung et al., 2010). Strains used in this study are listed in Table 1. AX2 and mutant strains were grown at 21 °C either on SM agar plates (ø 100 mm) with *Klebsiella aerogenes* (Williams and Newell, 1976) or in AX2 liquid nutrient medium in Erlenmeyer flasks with shaking at 160 rpm (Brink et al., 1990). In case of mutant strains the AX2 medium was supplemented with blasticidine (5 μg/ml) and/or G418 (6 μg/ml). For experimental use cells were harvested at a density of 2–4 × 10^6 cells/ml. For development on phosphate agar plates log phase cells from a shaking culture were washed twice with Soerensen buffer (14.6 mM KH_2PO_4, 2.0 mM Na_2HPO_4, pH 6.0). A total of 1 × 10^8 cells were then resuspended in 1 ml of Soerensen buffer and 500 μl of this solution corresponding to 5 × 10^7 cells was evenly distributed onto a phosphate-buffered agar plate (ø 100 mm) and incubated at 21 °C. To monitor developmental regulation of ATG8a and ATG8b

Table 1
D. discoideum mutant strains used in this study.

Strains	Summary	Reference
AAG	Expression of ATG9-GFP in the ATG9$^-$ background	Tung et al. (2010)
ATG9$^-$	Knockout mutant of ATG9	Tung et al. (2010)
AX2/[act15]:RFP-ATG8a	Expression of RFP-ATG8a in AX2	This work
AX2/[act15]:RFP-ATG8b	Expression of RFP-ATG8b in AX2	This work
ATG9$^-$/[act15]:RFP-ATG8a	Expression of RFP-ATG8a in ATG9$^-$	This work
ATG9$^-$/[act15]:RFP-ATG8b	Expression of RFP-ATG8b in ATG9$^-$	This work
AAG/[act15]:RFP-ATG8a	Expression of RFP-ATG8a in AAG	This work
AAG/[act15]:RFP-ATG8b	Expression of RFP-ATG8b in AAG	This work
AX2/[act15]:RFP-ATG8a/[act15]:GFP-ATG8a	Expression of RFP-ATG8a and GFP-ATG8a in AX2	This work
AX2/[act15]:RFP-ATG8a/[act15]:GFP-ATG8b	Expression of RFP-ATG8a and GFP-ATG8b in AX2	This work
ATG9$^-$/[act15]:RFP-ATG8a/[act15]:GFP-ATG8b	Expression of RFP-ATG8a and GFP-ATG8b in ATG9$^-$	This work